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Recovery and Altered Neutralization Specificities of Chimeric Viruses Containing Capsid Protein Domain Exchanges from Antigenically Distinct Strains of Feline Calicivirus

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Feline calicivirus (FCV) strains can show significant antigenic variation when tested for cross-reactivity with antisera produced against other FCV strains. Previous work has demonstrated the presence of hypervariable amino acid sequences in the capsid protein of FCV (designated regions C and E) that were postulated to constitute the major antigenic determinants of the virus. To examine the involvement of hypervariable sequences in determining the antigenic phenotype, the nucleotide sequences encoding the E regions from three antigenically distinct parental FCV strains (CFI, KCD, and NADC) were exchanged for the equivalent sequences in an FCV Urbana strain infectious cDNA clone. Two of the three constructs were recovered as viable, chimeric viruses. In six additional constructs, of which three were recovered as viable virus, the E region from the parental viruses was divided into left (N-terminal) and right (C-terminal) halves and engineered into the infectious clone. A final viable construct contained the C, D, and E regions of the NADC parental strain. Recovered chimeric viruses showed considerable antigenic variation from the parental viruses when tested against parental hyperimmune serum. No domain exchange was able to confer complete recognition by parental antiserum with the exception of the KCD E region exchange, which was neutralized at a near-homologous titer with KCD antiserum. These data demonstrate that it is possible to recover engineered chimeric FCV strains that possess altered antigenic characteristics. Furthermore, the E hypervariable region of the capsid protein appears to play a major role in the formation of the antigenic structure of the virion where conformational epitopes may be more important than linear in viral neutralization.

Feline calicivirus (FCV), a member of the family *Caliciviridae*, is frequently isolated from cats displaying acute upper respiratory disease and stomatitis as well as from cats that appear clinically normal. Antigenic relationships have been examined by serum neutralization (7, 21), plaque reduction neutralization (11), and monoclonal antibody (MAb) binding assays (14, 29, 30). These analyses have confirmed that there is significant antigenic variation among FCV strains. However, considerable cross-reactivity among strains has also been observed, based on two-way cross-neutralization tests (2, 11, 21). Thus, FCV strains have generally been considered as variants of a single serotype (11, 21).

Feline caliciviruses are nonenveloped viruses with diameters of 35 to 40 nm that contain a plus-sense, single-stranded, polyadenylated RNA genome. The viral shell is composed of 180 copies of a single capsid protein (22). The capsid protein is encoded within the 3′-terminal 2,400 bases of the genomic RNA and is translated primarily from the abundant 2.4-kb subgenomic RNA transcribed from this region (3, 9, 21, 30). The FCV capsid precursor protein ranges in size from 668 to 671 amino acids (aa) and shows an overall amino acid identity of 71%. The capsid precursor protein of the animal caliciviruses can be divided into six regions, A through F (19), based on the degree of amino acid conservation among FCV, San Miguel sea lion virus, and rabbit hemorrhagic disease virus. The A region, corresponding to aa 1 to 120 of the CFI strain of FCV, is highly conserved and is cleaved following synthesis in both FCV and San Miguel sea lion virus. Cleavage of the leader polypeptide has been shown to be mediated by a viral proteinase in FCV (28). An equivalent region is not present in rabbit hemorrhagic disease virus and the human caliciviruses. The B region (aa 121 to 396) contains sequences that are highly conserved among all the caliciviruses and is thought to form the viral core structure (19). The regions designated C (aa 397 to 411) and E (aa 426 to 521) were proposed to contain antigenic determinants of the virus because of sequence variability among antigenically diverse viruses (19, 24, 25). It was suggested that the observed cross-reactivity among FCV strains that resulted in the grouping of FCV strains into a single serotype may be related to the presence within the E region of a highly conserved amino acid core sequence of 31 aa. This core is flanked by two hypervariable sequences of 33 aa at the N terminus and 32 aa at the C terminus (25). The D region (aa 412 to 435) is highly conserved, and only minimal, conserved amino acid changes are observed in this domain of the capsid protein. The F region (aa 522 to 668) is found at the C terminus of the capsid protein and is moderately conserved among caliciviruses. The F region is thought to be at least partially exposed on the surface of the virion, based on the mapping of the binding site of a nonneutralizing MAb to this region (16).

MAb mapping experiments by Tohya et al. (29, 30) first demonstrated the presence of seven neutralizing epitopes on the FCV capsid. Studies by Guiver et al. (9), Milton et al. (16), and Shin et al. (26) subsequently mapped the binding sites of neutralizing MAbs to between amino acid residues 408 and 517 (entire E region), 422 and 458 (N-terminal half of the E region), and 381 and 454 (C and D regions and N-terminal half of the E region), respectively. These experiments indicated the

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TABLE 1. PCR oligonucleotide primer sequences

Primer[a]	Genomic location[b]	Sequence[c]
FCVE plus	6549–6578	TGTCCCTGGGATCCCTGATGGTTGGCCAGA
FCVE minus	6898–6929	GCTTCTTCTCCAATGCCAGTGTAGCCAAGGAG
NADC CDE plus	6166–6194	ACATCACTAGTAATTATGGTGTATAATGA
UrbPst plus	6719–6742	GTGGATCACTGCAGAGAGCGTGGG
UrbPst minus	6717–6740	CACGCTCTCTGCAGTGATCCACAG
NADCPst plus	6719–6742	GTGGTTCCCTGCAGAGAGCCTGGG
NADCPst minus	6717–6740	GCTCTCTGCAGGGAACCACAAATG
CFIPst plus	6719–6742	GTGGTTCTCTGCAGCTGAGCTTGGG
UrbBam plus	6715–6737	ATCTGTGGATCCCTCCAAAGAGC
UrbBam minus	6711–6733	TTTGGAGGGATCCACAGATATAC
KCDBam plus	6715–6737	GTTTGTGGATCCCTTCAAAGGGC
KCDBam minus	6711–6733	TTTGAAGGGATCCACAAACACAC
Urb plus[d]	6464–6483	GCACACCACGATTTAGACCA
Urb minus[d]	6959–6978	TTCGGGAAGCGTGCTGATGC

[a] Plus oligonucleotides prime plus-strand synthesis; minus oligonucleotides prime minus-strand synthesis.
[b] Nucleotide number in URB genomic RNA.
[c] Restriction endonuclease recognition sites are underlined.
[d] Sequencing primer for confirmation of E region exchange.

involvement of the E region in antigenicity and neutralization. Tohya et al. (31) confirmed these findings by the sequence analysis of MAb neutralization-resistant variants. Four linear and two conformational epitopes were identified in the two hypervariable portions of the E region. In all cases, the resistance to MAb neutralization was associated with a single nucleotide change that resulted in an amino acid substitution. The six amino acid substitutions identified fell within the regions identified in the earlier MAb mapping experiments. Kruetz et al. (14) analyzed the sequence of the E regions in viruses isolated from persistently infected cats (10), some of which showed significant differences in antigenicity from the original infecting strain. This study revealed that relatively minor amino acid changes in the variable portions of the E region had a profound effect on antigenicity. Many of the amino acid changes occurred within the regions of the E region identified by Tohya et al. (31).

The goal of this study was to examine the role of the hypervariable E region in determining the antigenic phenotype of the FCV virion. An infectious cDNA clone of the Urbana (URB) strain of FCV (27) was used to examine the effect of replacement of the hypervariable sequences of the URB capsid protein with those from antigenically distinct FCV strains. The parental strains used here (CFI, KCD, and NADC) were chosen because of the distinct serologic differences reported previously (11, 21, 25). It was not known whether the FCV capsid protein could tolerate such domain exchanges, but the recovery of viable, chimeric viruses would allow an analysis of the effects of these exchanges on virus neutralization.

MATERIALS AND METHODS

Cells, viruses, and RNA purification. FCV parental strains CFI (5), KCD (8), NADC (24), and URB (27) and chimeric viruses were propagated in Crandell-Rees feline kidney (CRFK) cells as previously described (17). The CFI and KCD strains were obtained from the American Type Culture Collection (Manassas, Va.). Total cellular RNA from FCV-infected cells was prepared by the guanidine-acidic phenol method (4) and was used for reverse transcriptase-mediated PCR of capsid sequences for domain exchanges and for sequence analysis of exchanged regions in chimeric viruses. Viruses used for antiserum production were grown in roller bottles and purified by CsCl isopycnic centrifugation (17).

Plasmids. Plasmid pFI-28, which contained the 3′-terminal 4,657 bp of the URB strain of FCV (27), was modified to serve as a shuttle vector for replacement of nucleotide sequences of the FCV capsid protein gene. pFI-28 was digested with *Sma*I, which cuts the single *Sma*I site in the polylinker of the pSPORT1 plasmid. This was followed by ligation of *Spe*I synthetic linkers to the ends and digestion with *Spe*I, which digested the linkers as well as a *Spe*I site at nucleotide 3084 of the URB clone. The plasmid was recircularized by ligation. A plasmid containing only the 3′ terminal 1,573 bases of the URB genome was designated pFI-28spe and was used in all further DNA manipulations. This plasmid contained single *Spe*I, *Not*I, *Msc*I, and *Sty*I restriction sites that were used in further plasmid constructs. The latter two restriction sites flanked the E region sequences of the capsid protein gene and allowed replacement of this domain.

Primers and PCRs. PCRs were performed as previously described (20) with the exception that *Taq* DNA polymerase was replaced with Expand high-fidelity polymerase mix (Boehringer Mannheim, Inc., Indianapolis, Ind.). Primers were designed using capsid gene sequences from the CFI, KCD, NADC, and URB sequences (GenBank accession no. M32819, L09718, L09719, and L40021, respectively). A cDNA clone of each parental capsid protein gene was used as the template in amplification reactions. Each parental virus E region was amplified by using FCVE plus and FCVE minus primers (Table 1). To amplify the C, D, and E regions in a single fragment, the FCVE minus primer was used with the NADC CDE plus primer. Half-site domain exchanges, involving only one half of the E region, were generated by using primers that annealed to the highly conserved central core sequences of the E region of FCV. These primers were designed to create new restriction sites without altering the amino acid sequence encoded by the DNA fragment. The half E region was amplified from the donating strain with the other half E region amplified from the URB strain. The two fragments were purified by using GeneClean resin as specified by the manufacturer (Bio 101, Inc., Vista, Calif.). The fragments were digested with the appropriate restriction endonuclease (*Bam*HI for the KCD half site or *Pst*I for the NADC and CFI half-site), pooled and ligated in a 50-μl total volume. The full-length E region was amplified by using 2 μl of the ligation reaction mixture and the FCVE plus and FCVE minus primers in a standard PCR.

Cloning of PCR products. Following amplification, the single DNA fragment from the E regions, CDE region, and half-site exchanges were purified from the PCR products by using GeneClean resin. The E region and half-site exchange fragments were digested with *Msc*I and *Sty*I and were ligated into *Msc*I/*Sty*I-digested pFI-28spe. The CDE region exchange was digested with *Spe*I and *Sty*I and ligated into *Spe*I/*Sty*I-digested pFI-28spe. Recovered plasmids were screened for the presence of restriction sites present in the exchanged fragment and not in the original URB sequences. The plasmids were then sequenced to confirm the fidelity of the sequence.

Construction and recovery of chimeric viruses. The confirmed domain exchanges constructed in pFI-28spe were transferred into the FCV URB infectious cDNA clone pQ14 (27) by digestion of the exchange-containing plasmid with *Spe*I and *Not*I and subsequent ligation of the chimeric FCV sequences into *Spe*I/*Not*I-digested pQ14. Recovered plasmids were screened for the proper insert with restriction endonuclease digestion and were sequenced to confirm the fidelity of exchanged sequences. RNA transcription and transfection of synthetic, capped RNA into susceptible cells were performed as previously described (27). Virus recovered following transfection was plaque purified three times and analyzed for the domain exchange by PCR amplification of the E region and restriction digestion of the PCR product with a differentiating restriction endonuclease. Following recovery of virus, CRFK cells were infected, and culture fluids containing virus were frozen at −80°C until further use. The nomenclature and domain exchanges for each construct are shown in Table 2 and Fig. 1.

Virus titration. Viruses were titrated by using serial 10-fold dilutions of virus in a 96-well microtiter plate format. Virus dilutions (five replicates) were placed in wells, and CRFK cells were added to each. The plates were incubated at 37°C in 5% CO_2 atmosphere for 48 h. Viral titers were calculated by the method of Reed and Muench (23).

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TABLE 2. Chimeric capsid protein constructs[a]

Virus	Domain exchanged	Viable virus recovery
U/N_E	NADC E region	Yes
U/K_E	KCD E region	Yes
U/C_E	CFI E region	No
U/N_{EL}	NADC N terminus of E region URB C terminus of E region	Yes
U/N_{ER}	URB N terminus of E region NADC C terminus of E region	Yes
U/K_{EL}	KCD N terminus of E region URB C terminus of E region	No
U/K_{ER}	URB N terminus of E region KCD C terminus of E region	No
U/C_{EL}	CFI N terminus of E region URB C terminus of E region	Yes
U/C_{ER}	URB N terminus of E region CFI C terminus of E region	No
U/N_{CDE}	NADC C, D, and E regions	Yes

[a] The E region includes amino acid residues 426 through 521; the N terminus of the E region includes amino acid residues 426 through 470; the C terminus of the E region includes amino acid residues 471 through 521; the C, D, and E regions include amino acid residues 397 through 521. Subscripts L and R in virus names represent left and right, respectively.

Antiserum and virus neutralization assays. Antisera were raised against FCV parental and chimeric viruses by emulsification of CsCl-purified viruses in TiterMax adjuvant (CytRx Corp., Norcross, Ga.) and injection of female New Zealand White rabbits as recommended by the manufacturer. Neutralization titers (NT) of each antiserum were determined for homologous and heterologous viruses by the virus neutralization test using the 96-well microtiter plate format. Briefly, antisera were diluted serially twofold and mixed with 100 50% tissue culture infective doses of virus. Each serum dilution was tested in five replicates. After incubation at 37°C for 1 h, CRFK cells were added to each well and the plate was incubated at 37°C in 5% CO_2 for 48 h. Each well was examined for cytopathic effect, and the antibody titer was determined to be the highest antiserum dilution that gave complete neutralization in all five test wells. All virus-antiserum combinations were tested in at least two independent tests.

The Archetti and Horsfall analysis (1) was used to compare the antigenic relatedness of the parental and chimeric viruses following two-way cross-neutralization analysis. Briefly, the equation $r = \sqrt{r_1 \times r_2}$, where r is the geometric mean of the titer ratios r_1 and r_2, was used. 1_1 was calculated by dividing the titer of the antiserum to virus 1 against virus 2 by the titer of antiserum to virus 1 against virus 1. 1_2 was calculated by dividing the titer of the antiserum to virus 2 against virus 1 by the titer of the antiserum to virus 2 against virus 2. An r value of ≤ 0.5 or ≥ 2 indicated a significant difference in antigenicity between two viruses. This analysis was limited to the chimeric viruses for which antisera were available.

RESULTS

Chimeric virus recovery. Following transfer of the URB sequences containing the E region exchanges into pQ14, full-length capped RNA transcripts were generated and used to transfect CRFK cells. Viable chimeric viruses were recovered for all exchanges with the exception of the CFI E region, the CFI E right (C-terminus) half site, and both KCD half-site exchanges (Table 2). Sequence analysis of the four recombinant plasmids that did not yield viable progeny showed the exchanged regions and the regions adjacent to these sequences to be correct, with no frameshift or premature termination codons. However, we did not determine whether point mutations in other regions of the genome were introduced during cloning. Other possible reasons for failure to recover these chimeric viruses include (i) unfavorable interactions between regions of the capsid protein necessary for assembly and stability of the virus particles and (ii) disruption of interactions of the chimeric capsid protein with other viral proteins or RNA. Additional studies into the mechanisms responsible for the failure to recover these chimeric viruses are in progress.

Neutralization specificity of parental antisera against parental viruses. Specific antisera produced in rabbits against the four parental strains URB, KCD, NADC, and CFI were used to compare differences in antigenicity by virus neutralization tests (Table 3). The antisera (Table 3) showed homologous NTs ranging from 1:8,192 (URB) to 1:65,536 (KCD and NADC). The heterologous NTs of the parental hyperimmune sera ranged from 1:32 (anti-KCD versus URB and CFI) to 1:1,024 (anti-NADC versus KCD).

Cross-neutralization data for the parental strains were analyzed by the stringent Archetti and Horsfall test. The antigenic difference is considered significant when the r value is ≥ 2. The r values obtained from the parental strain NTs ranged from 184 (anti-URB versus CFI) to 525 (anti-KCD versus CFI [data not shown]).

Neutralization specificity of parental virus antisera against chimeric viruses. The NTs of the parental antisera against the chimeric viruses were, in most cases, significantly different from those observed against the parental viruses (Table 3). Of interest, the URB antiserum did not neutralize the chimeric viruses efficiently, although the capsid protein was derived primarily from the URB strain. All chimeric viruses showed decreases of 8- to 64-fold in NTs from the URB homologous NT. The NTs with KCD antiserum showed no specific recognition of the chimeric viruses with the exception of the chimeric virus U/K_E, which was neutralized at 1:32,768, an NT that was similar to the homologous NT of this antiserum with KCD (1:65,536). This was the only observed example of efficient recognition of a chimeric virus by a parental antiserum. The parental CFI antiserum did not neutralize the recovered chimeric viruses efficiently, although there were elevated NTs against the CFI chimeric virus U/C_{EL} (1:1,024), as well as U/K_E (1:1,024) and U/N_{CDE} (1:512), compared to the NT of 1:64 for this serum against URB (8- to 16-fold higher).

The NADC antiserum showed elevated NTs against three of the four chimeras that contained NADC sequences compared to the NT of anti-NADC versus URB (1:128). The U/N_E, U/N_{EL}, and UN_{CDE} viruses had NTs that were at least 16-fold higher (1:2,048, 1:2,048, and 1:8,192, respectively), while the U/N_{ER} was 4-fold higher (1:512). The NT for U/N_{ER} was at least fourfold less than the NT for the other three NADC-containing chimeric viruses, indicating that U/N_{ER} was less closely related antigenically to the parental NADC strain. In contrast to U/K_E, which was neutralized by KCD antiserum, the U/N_E chimeric virus was not neutralized at a high serum dilution by NADC antiserum.

Neutralization specificity of antisera raised against chimeric viruses. Antisera raised against three chimeric viruses, U/N_E, U/N_{EL}, and U/K_E, showed high homologous NTs (Table 3) and demonstrated marked specificity for the immunizing virus. All homologous titers of the chimeric virus hyperimmune sera were at least 16-fold higher than with any other virus tested, including the parental strains. The lack of efficient neutralization of KCD by U/K_E antiserum was of interest because of the efficient neutralization of U/K_E by the KCD antiserum.

The three chimeric viruses against which antiserum was raised were shown to be antigenically distinct from each other as well as the parental viruses by the Archetti and Horsfall method. The r values obtained from this analysis ranged from 14 to 533 (data not shown).

DISCUSSION

An FCV infectious cDNA clone was used in this study to construct and recover chimeric progeny viruses containing exchanged nucleotide sequences encoding the hypervariable se-

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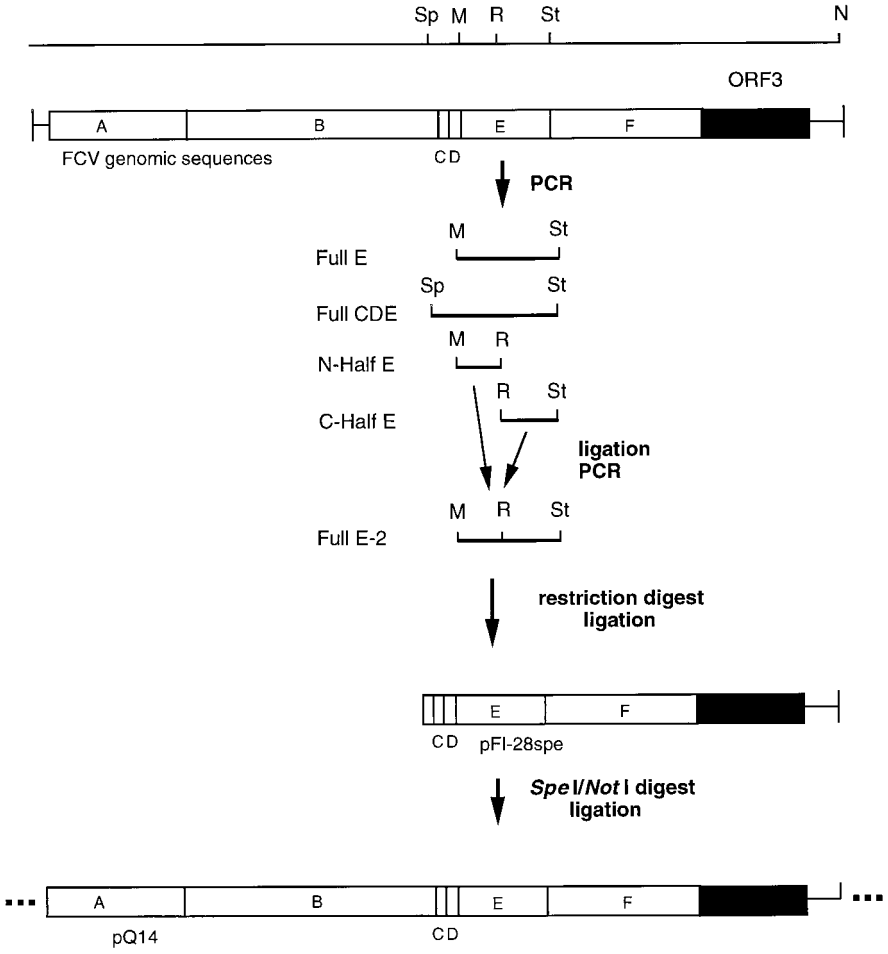

FIG. 1. PCR amplification and subcloning of E region sequences from the FCV capsid protein gene to construct domain exchange chimeric viruses. The domains of the FCV capsid protein are shown at the top as encoded by the 2.4-kb subgenomic RNA. A to F represent previously described domains of the capsid protein (17). ORF3 is a small open reading frame immediately following the capsid protein gene. The sequences encoding the domain to be exchanged were PCR amplified with restriction sites included on the ends. The line above the genomic sequences show locations of the restriction sites used in the DNA manipulations. Sp, M, St, and N represent *Spe*I, *Msc*I, *Sty*I, and *Not*I restriction sites, respectively, found in the URB cDNA sequences; R represents a restriction site engineered to construct the half-domain exchanges. The full E domain contains the complete E region plus some flanking sequences. The full CDE domain contains the complete C, D, and E regions plus some flanking sequences. The N-half E (N-terminal) and C-half E (C-terminal) domains were used to construct the half-site exchanges. The half E fragments were digested with the R restriction endonuclease, ligated, and reamplified as with the full E domain to yield the full E-2 domain. The E, E-2, and CDE domains were cloned into the URB strain FCV pFI-28spe shuttle vector and then into the pQ14 URB strain infectious cDNA clone (25) as a *Spe*I/*Not*I fragment. The three periods at the ends of the sequences represent the remainder of the plasmid.

quences of the single capsid protein. A total of six chimeric viruses were recovered and characterized. Transfer of the capsid protein hypervariable E region sequences into the URB strain from antigenically distinct FCV strains had a dramatic impact on the antigenicity of the recovered chimeric viruses. In general, the chimeric viruses showed antigenic differences from the URB strain that appeared as 8- to 32-fold-lower titers against the URB hyperimmune serum, even though the URB

TABLE 3. Cross-neutralization titers of parental and chimeric FCVs by rabbit anti-FCV hyperimmune serum

Serum	NT[a]									
	URB	NADC	KCD	CFI	U/N_E	U/K_E	U/N_{EL}	U/N_{ER}	U/C_{EL}	U/N_{CDE}
URB	***8,192***	*256*[b] (32)	*128* (64)	*128* (64)	256 (32)	256 (32)	1,024 (8)	512 (16)	1,024 (8)	512 (16)
NADC	*128* (512)	***65,536***	*1,024* (64)	*64* (1,024)	2,048 (32)	4,096 (16)	2,048 (32)	512 (128)	256 (256)	8,192 (8)
KCD	*32* (2,048)	*64* (1,024)	***65,536***	*32* (2,048)	64 (1,024)	32,768 (2)	64 (1,024)	16 (4,096)	64 (1,024)	128 (512)
CFI	*64* (512)	*128* (256)	*256* (128)	***32,768***	128 (256)	1,024 (32)	64 (512)	32 (1,024)	1,024 (32)	512 (64)
U/N_E	128 (512)	1,024 (64)	256 (256)	128 (512)	**65,536**	2,048 (32)	2,048 (32)	512 (128)	2,048 (32)	2,048 (32)
U/K_E	128 (2,048)	32 (8,192)	2,048 (128)	128 (2,048)	256 (1,024)	**262,144**	256 (1,024)	256 (1,024)	1,024 (256)	512 (512)
U/N_{EL}	1,024 (64)	2,048 (32)	512 (128)	256 (256)	512 (128)	128 (512)	**65,536**	512 (128)	2,048 (32)	4,096 (16)

[a] Reciprocal of highest dilution giving complete neutralization. Boldface indicates homologous NT; italicized values are NTs obtained for parental viruses versus parental hyperimmune serum; values in parentheses represent fold decrease of NT of heterologous virus from homologous virus.
[b] NT of heterologous virus.

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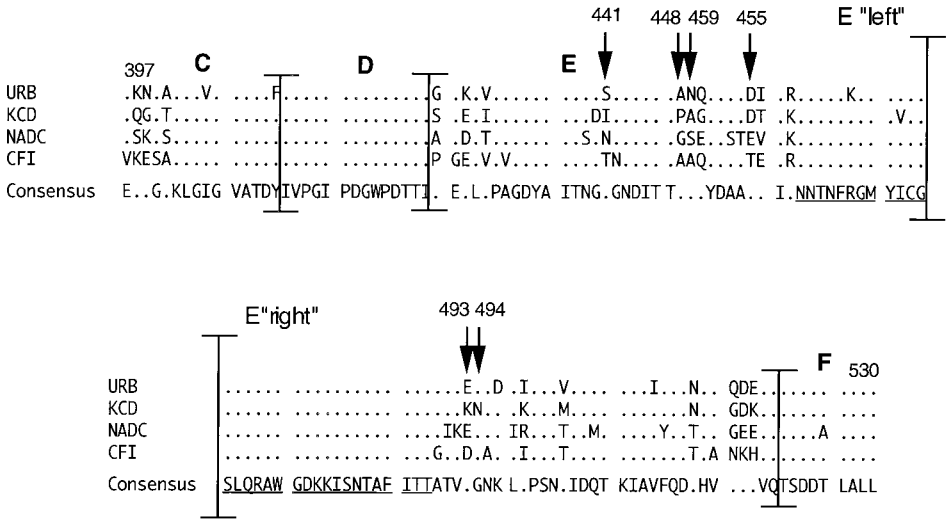

FIG. 2. Alignment of amino acid sequences of the C, D, and E domains of the parental strains CFI, KCD, NADC, and URB. The domains are marked by vertical lines, as is the border between the left and right halves of the E region. The amino acid residues are numbered from the start of translation of the capsid protein of the URB strain. Arrows indicate the amino acid residues that were identified as changed by Tohya et al. (29) in MAb neutralization-resistant variants of FCV. The underlined residues are those that make up the highly conserved central core region.

sequences comprised from 64 to 92% of the capsid protein. Thus, major URB antigenic traits were lost following the introduction of the heterologous capsid sequences. However, in general, the exchanged sequences were unable to confer complete antigenic traits of the donor parent to the chimeric viruses. The only exception was the U/K_E chimera. In this case, the exchanged KCD E region conferred an antigenic phenotype that was efficiently recognized by the KCD antiserum. However, the antiserum raised against the U/K_E virus failed to efficiently neutralize the URB and KCD parental viruses as well as the other heterologous viruses. The two other chimeric viruses containing complete E region exchanges (U/N_E and U/N_{CDE}) were not neutralized efficiently by the parental NADC antiserum, nor did U/N_E antiserum efficiently neutralize NADC. Thus, the reason for the efficient recognition of U/K_E by the KCD antiserum is unclear. It is possible that an intact KCD antigenic site was transferred into the URB capsid that maintained an authentic conformation recognized by the KCD antiserum. However, the inability to transfer such a site from the NADC strain into the URB capsid suggests that the conformation of the capsid, and not the primary amino acid sequence, is the major determinant of antigenic specificity.

Antisera raised against the chimeric viruses appeared to have, in many cases, NTs that were greater against other chimeric viruses than the NTs against parental viruses. The only apparent connection among the chimeric viruses was the shared URB sequence contained in the capsid proteins. Conservation of this sequence undoubtedly resulted in the generation of common antigens among the chimeric viruses.

The majority of chimeric viruses successfully recovered contained sequences derived from the NADC parental strain. Thus, this set of chimeric viruses allowed the greatest number of comparisons to be made between two parental viruses. Although preliminary, our data suggest that there may be a difference in the degree of recognition conferred by the two halves of the E region. Comparison of the NTs for the U/N_{EL} and U/N_{ER} viruses showed that the parental NADC antiserum recognized the U/N_{EL} at a fourfold-higher titer than it recognized the U/N_{ER} virus. In contrast, the URB antiserum recognized U/N_{ER} at a slightly (twofold) higher titer than U/N_{EL}. These data suggest that the left (N-terminal) portion of the E region may play a slightly greater role in antigenic specificity. This possibility is further supported by the finding that the majority of the neutralizing MAbs map to this portion of the E region (Fig. 2) (8, 14, 24). Inclusion of the variable C region in the U/N_{CDE} chimera had only a minimal change in the degree of recognition by NADC antiserum, with a fourfold increase in NT of U/N_{CDE} over U/N_E. The 1:8,192 NT of U/N_{CDE} was still eightfold lower than the homologous NADC NT. The U/N_{CDE} chimera contained the largest amount of exchanged capsid sequences, yet it did not assume full serologic identity with the NADC parent.

An interesting observation was the general inability to recover chimeric viruses with CFI E region exchanges. The URB capsid protein could tolerate only the CFI E left region. A comparison of the amino acid sequences of the C, D, and E regions of the parental viruses (Fig. 2) found that 17 of the amino acid residues were hypervariable among all four parental viruses, based on the inability to form a consensus at that residue. Of these 17 residues, 4 were identical between URB and CFI in the left portion of the E region. Only one such conserved hypervariable amino acid was conserved between URB and CFI in the right half. The higher conservation of hypervariable amino acids in the left half of the E region may have been sufficient to confer the ability to recover the U/C_{EL} chimera. The arrows in Fig. 2 indicate the six amino acid residues identified by Tohya et al. (31) that were changed in the MAb neutralization-resistant variants. Five of these six residues were hypervariable in all parental viruses.

The data presented here support the observation that the larger the region exchanged, the greater the degree of recognition by the E region donor parental antisera. The general inability to confer complete or near complete recognition by parental antisera to the chimeric viruses illustrates that some portion of the antigenic determinants that play an additional role in antigenicity may be missing from these chimeric viruses. For example, the moderately conserved F region has been shown to have at least some surface exposure on the virus particle (16). The involvement of the F region sequences was not investigated here. The lack of recognition of parental vi-

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ruses by the chimeric antiserum (and vice versa) demonstrates that linear epitopes may play only a minor role in antigenicity and neutralization. Taken together, our data suggest that conformational epitopes formed by the interaction of sequences from different regions of the capsid protein or between neighboring capsid proteins play the major role in determining the overall antigenic phenotype of the virus. This may explain why relatively minor changes in amino acid sequence (14) can cause dramatic changes in antigenicity. A minor change in amino acid sequence may be sufficient to disrupt the antigenically important conformational structure. Further structural studies are needed to address the role of intra- and intercapsid protein interactions in determining antigenic specificity.

The neutralization test has been used to define distinct serotypes for a number of different viruses, with the criterion of a reciprocal >20-fold difference in neutralizing antibody titer between a candidate strain and an established serotype (6, 12). Our results indicate that the four parental FCV strains examined in this study meet this criterion. In earlier FCV studies, antigenic relationships were established by using antisera obtained from cats undergoing natural infection or raised in large animals injected with the virus. It is possible that these approaches produced antisera that were not uniformly serotype specific. For example, in two earlier studies by Povey (21) and Kalunda et al. (11), antisera were raised against the KCD FCV strain in goats; however, Povey determined KCD to be what he called a distinct type, while Kalunda et al. found it to be broadly reactive. In addition, these two studies and others showed extensive cross-reactivity among FCV strains with sera from cats undergoing natural infection with FCV; thus, all FCV strains have been considered as belonging to a single serotype (11, 21). In this study, hyperimmune sera were raised in rabbits by an immunization strategy similar to that used in other virus systems to generate antisera with serotype-specific neutralization specificity (13). The testing described here was not designed to develop an FCV serotyping scheme. However, it does indicate that distinct FCV serotypes exist and that additional testing with standardized antisera may be useful in determining whether serotypic diversity plays a role in the natural history of FCV.

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